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(54) Title: SEQUENCING METHOD

(57) Abstract

A method of sequencing a single-stranded nucleic acid chain involves providing an immobilised complex of a template to be sequenced and a primer. This complex is exposed to flow containing only one dNTP at a time. The primer is extended only if the dNTP is complementary to and can hybridise to the next free nucleotide of the template. Each such polymerisation event is detected, preferably directly and by spectroscopic means. Preferred detection techniques include fluorescence detection and absorption spectroscopy (non-evanescent wave) and particularly evanescent spectroscopy e.g. surface plasmon resonance spectroscopy.

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SEQUENCING METHOD

5   Introduction

Determination of the sequence of nucleotide
bases in 2'-deoxyribonucleic acid (DNA) and to a lesser
extent ribonucleic acid (RNA) is a central technology
for much of present day molecular biology and its many
10 applications.   There are currently two main methods
available for sequencing DNA, and both involve using a
DNA polymerase enzyme to make a copy of a suitably
primed strand of the unknown DNA which acts as a
template.   In the so-called chain-termination method
15 of Sanger et al (1977), the growing copy strand is
randomly (at least, randomness is intended) terminated
by incorporation of a 2', 3'-dideoxyribonucleotide
(ddNTP) competing with the normal 2'-
deoxyribonucleotide (dNTP) for addition to the 3'-end
20 of the growing copy strand.   By using suitable
conditions and four separate preparations corresponding
to dATP with ddATP, dCTP with ddCTP, dGTP with ddGTP
and dTTP with ddTTP, all possible lengths of terminated
copy strands are available.   They are separated by
25 size by gel electrophoresis, and detected by means of
an incorporated label, commonly radioactive but more
recently fluorescent (Smith et al, 1986; Ansorge et al,
1987).   The DNA sequence of the copy, and therefore
the complementary sequence of the template, is derived
30 from the pattern of chain lengths detected for the
separate A, C, G and T termination mixture.

The chemical degradation method of Maxam and
Gilbert (1977) also uses DNA polymerase catalysed
formation cf a template-copy.   The dNTP's are radio-
35 active with ^{32}P (or ^{35}S) at the α-position, so the copy
is also radioactive.   Chemical treatments specific for

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different bases cleave the copy into sets of fragments
which, as with the Sanger dideoxy chain termination
method, can be separated by gel electrophoresis and
detected (by autoradiography) to yield patterns from
5   which the DNA sequence can be deduced. · Variants of
these methods have been described (Church & Kieffer-
Higgins, 1988; Gish and Eckstein, 1988), but are still
dependent upon gel electrophoresis to separate DNA
fragments, and upon such methods such as radiolabelling
10   for detection of the separated fragments.

All of these methods are slow, complex and
expensive in relation to the needs for DNA sequencing.
For example, the best rates of DNA sequencing to date
are about 600 nucleotides in 12 hours, using an
15   automated instrument (Landegran et al, 1988). This
corresponds to 50 bases per hour or 0.014 bases per
sec. This rate is approximately two-orders of
magnitude lower than that thought necessary for large-
scale sequencing in the Human Genome Project (Alberts
20   et al, 1988).

Three laboratories have reported attempts to
avoid the needs for copy-strand fragments and their
length-determination by gel electrophoresis. Hyman
(1988) immobilised the DNA template primer with
25   polymerase on an ion-exchange material and exposed the
complex to a flow wherein only one of the four dNTP's
was present at a time. A downstream detection system
then detected the pyrophosphate liberated in the DNA
polymerase reaction:-
30   template - primer + dNTP -> template - (primer + dNMP) + PPi
where dNMP stands for the added nucleotide residue, PPi
for pyrophosphate. The sequencing rate was
approximately 1 base every 10 minutes. Melamede
(1987) also proposed the use of an immobilised DNA
35   template, primer and polymerase complex exposed to a
flow containing only one species of dNTP at a time.

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However, incorporation of dNTP into the copy was
calculated not from measurements of pyrophosphate
release but from the difference in dNTP concentrations
entering and leaving the flow cell which contains the
5   complex of DNA template and polymerase. No
experimental data were presented.

Jett (1989) provided a single stranded DNA or
RNA sequence of labelled nucleotides, complementary to
the sequence to be determined, suspended in a moving
10 flow stream. An exonuclease sequentially cleaved
individual basis from the end of the suspended
sequence. The resulting train of individual labelled
nucleotides was passed to a downstream location for
analysis of the individual nucleotides.

15 This invention

As with the methods of Melamede (1987) and
Hyman (1988), we also use an immobilised complex of
template and primer, exposed to a flow containing only
one dNTP at a time. We differ however in that we
20 directly measure the growth of the template copy,
rather than infer it indirectly from changes in the
composition of the flow medium. The methods of
detection are preferably spectroscopic and do not
require the further addition of chemical reagents to
25 effect measurement of template-copy growth. The
spectroscopic methods are:-
i)   Evanescent wave spectroscopy
ii) Fluorescence detection (non-evanescent wave)
iii) Absorption spectroscopy (non-evanescent wave)

30   Alternatively the individual nucleotides may
be labelled, e.g. radioactively. Attachment of each
successive labelled nucleotide to the immobilised
complex is then detected by an increase in total
radioactivity.

35   Irrespective of which detection method is
used, the time-dependent signal arising from

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polymerase catalysed template copy growth depends on
the availability of the correct (i.e. complementary)
nucleotide for base-pairing with the template at the
growing point of the copy.   If all four nucleotides
5 are absent, there is no polymerase activity. If only
one nucleotide is present, then extension of the copy
will occur only as far as base-pairing with the
template is possible in the absence of the other three
nucleotides.

10   Preferably a continuous flow of pulses of
deoxynucleotides (dATP, dCTP, dGTP, dTTP) or
ribonucleotides (ATP, CTP, GTP, UTP)or analogues of
these nucleotides, each separated by a wash pulse, is
passed over the tethered template DNA or RNA (with any
15 extension and linkers, and the primer oligonucleotide).
This is done under conditions, e.g. in the presence of
a suitable polymerase enzyme, to cause an extension of
the primer by addition of the nucleotide (or analogue)
to the 3'-end of the primer if the nucleotide (or
20 analogue) is complementary to and can hybridize with
the next free nucleotide of the template (going from
the 3'-end towards the 5'-end). Conditions of solvent,
temperatures, ionic strength and concentrations of any
polymerase enzyme activating cations and anions are
25 maintained such that the polymerase is enzymatically
active in the presence of its substrates. Because
only one of the four nucleotides is present at a time,
extension of the copy strand occurs in jumps depending
on whether the appropriate nucleotide for pairing with
30 the next unpaired base of the template strand is
present or not. Each such jump is herein designated a
polymerisation event. Thus the signal also exhibits
time-dependent jumps that reflect the extension jumps
(polymerisation events) of the copy strand.
35 Furthermore, the size of the signal jumps is
proportional to the number of bases added in each

extension jump (i.e. proportional to the number of
polymerisation events), e.g. the addition of say three
adenine residues gives three times the signal given by
the addition of only one such residue.   Because the
5   copy strand can be extended by only an integral number
of bases (0, 1, 2, 3...) during any extension jump,
then the optical signals are quantised.   This property
of the signals is highly advantageous for calibration
and for discrimination against noise.
10   For simplicity, this invention is hereafter
described in terms of DNA sequencing, using a DNA
template with DNA polymerase and deoxynucleotides
(dNTP's) or analogues to make a DNA copy.   It should
be noted that RNA technologies could be used : e.g. (i)
15   DNA as the template, RNA polymerase to make an RNA copy
(ii) RNA as the template, reverse transcriptase to make
a DNA copy, and (iii) RNA as the template, RNA
replicase to make an RNA copy.

(i)   Evanescent wave spectroscopy (EWS)

20   Evanescent wave spectroscopy is defined in
the present context as embracing three related methods:
(i) attenuated total reflection (ATR) spectroscopy (ii)
total internal reflectance fluorescence (TIRF)
spectroscopy, and (iii) surface plasmon resonance (SPR)
25   spectroscopy.   Each of these three spectroscopic
techniques examines an optical property of a solution
bordering a surface where total internal reflection of
a light beam has occurred.   In each case the incident
and reflected beams are on the side of the surface
30   distal to (i.e. remote from) the solution under study,
whereas the evanescent wave is established on the
solution side of the surface but extends into that
solution for a very short distance, typically less than
the wavelength of the incident/reflected beam.
35   Spectroscopy by ATR or TIRF requires only a transparent
material such as glass or quartz to create the

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interfacial surface with the solution, whereas SPR
spectroscopy requires that the glass or quartz surface
be coated with a thin (e.g. 50nm) metal layer of, for
example, silver.   All three methods can detect the
5 exchange of solute molecules between the bulk phase of
the solution and the interfacial surface, albeit by
different means.   ATR spectroscopy detects the
absorption of evanescent wave light by molecules, with
appropriate absorption spectra, that lie within the
10 evanescent wave region.   If the absorbed light is re-
emitted as fluorescence then the emission can be
measured with a suitable detector, such as a photo-
multiplier tube, leading to TIRF spectroscopy.   Thus
both ATR and TIRF spectroscopy measure the absorption
15 of light by molecules at or close to the interfacial
surface, the difference being that ATR measures the
absorption directly whereas TIRF measures it
indirectly, as re-emitted fluorescence.   By contrast,
SPR spectroscopy measures changes of refractive index
20 that may occur in the SPR evanescent wave region, but,
just as with ATP and TIRF, those changes arise due to
redistribution of molecules between the bulk phase of
the solution and the evanescent wave region.   For a
review of these methods, see Sutherland and Dahne,
25 1987.

        Having given a brief outline of evanescent
wave spectroscopies, we can describe their application
to DNA sequencing.   The DNA molecule of unknown
sequence, derived from biological techniques such as
30 DNA cloning or polymerase chain reactions must (i) be
single stranded (ii) be attached either directly or
indirectly by either its 3' or 5' end to the
interfacial surface of an EWS device, and (iii) possess
a known sequence, either naturally occurring or added,
35 at its 3' end such that a complementary oligonucleotide
(primer) can be hybridised to create a run of double

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stranded DNA.   Thus in the presence of DNA polymerase
and deoxyribonucleotide triphosphates (dNTP) the
unknown DNA acts as a template for the synthesis of a
complementary copy extending by growth from the 3' end
5 of the primer. DNA polymerase catalysed growth of the
template copy results in recruitment of dNTP's from the
bulk phase to the interfacial region where the template
is tethered. It is assumed that the copy strand
remains base-paired to the template strand, and does
10 not diffuse away. Template copy growth can therefore
be measured by EWS spectroscopy. ATR spectroscopy
requires that the incident beam be in the wavelength
region of a major absorption band of the dNTP's,
typically about 260nm. TIRF spectroscopy requires that
15 the nucleosides incorporated into the template copy can
be excited to fluoresce by the evanescent wave :
fluorescent DNTP analogues are required. SPR
spectroscopy has fewer limitations : dNTP analogues are
not required (although substitution with refractive
20 index enhancing atoms such as Br, I or Hg might be
advantageous for sensitivity) and the choice of
wavelength is not critical.

Figure 1 illustrates the experimental set up,
shown for the specific case of SPR spectroscopy.   The
25 3' end of the template DNA (or its extension) is
attached to the interfacial silver surface, and the
primer oligonucleotide is hybridised to the template.
Alternatively the primer could be attached to the
surface and the template DNA hybridised to the primer.
30 Clearly the polarity of attachment can be reversed:-
the 5' end of the template bound to the surface, and
the primer hybridised to the free 3' end of the
template or extension.

Figure 2 shows idealised experimental results
35 using SPR spectroscopy. The sequence of the template
being copied is read directly from the associations

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between jumps in the EWS signal and the particular dNTP
pulse present at the template site.

(ii) Fluorescence detection

Because each cycle of dNTP (or rather its
fluorescent analogue) is preceded and followed by a
wash, EWS (TIRF) is not mandatory to distinguish the
fluorescence signal arising from the template-copy from
free nucleotides in the medium.   Thus a more
conventional optical geometry can be used, and
immobilisation of the template-primer-polymerase
complex can be on beads, fibres or membranes rather
than an evanescent wave surface.

A serious drawback of the fluorescent method
for DNA sequencing, irrespective of whether or not it
uses an evanescent wave, is the fact that the absolute
size of the signal grows as the copy strand grows.
This makes it harder to detect the increments (jumps)
of fluorescence resulting from strand extension.

Even under ideal conditions (Poissonian
photon-counting statistics, zero background and
noise-less excitation source), it is necessary to
increase the measurement time for each increment of
nucleotide addition in proportion to the number of such
increments in order to maintain constant sensitivity
for the detection of each successive fluorescence
increment.   For example, if it took 1 sec to measure
the fluorescence of the first added nucleotide to a
satisfactory accuracy, then 10 seconds would be needed
to measure the tenth added nucleotide, 100 seconds for
the hundredth, and so on.   Thus what is intended to be
a fast DNA sequencing method becomes progressively
slower as it proceeds.

There are two instrumental solutions to this
problem.   The first is to increase not the duration of
measurement for successive increments of fluorescence,
but the excitation intensity.   By this means the

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accumulation of photon counts required for accuracy can
be achieved in a constant time.   Technically this can
be arranged with a CW laser excitation source equipped
with an acousto-optic or electro-optic modulator to
5   modify the beam intensity transmitted to the sample.
The second solution is to irreversibly photobleach the
sample fluorescence with an intense light pulse at
periodic intervals, thereby resetting the sample
fluorescence to zero before it can significantly
10   degrade the detection of successive increments.
Technically this can also be achieved with a CW laser
equipped with a modulator (Garland, 1981).
         The fluorescent probe itself can be attached
to each of the four dNTP's.   As each dNTP is presented
15   separately to the template-primer-polymerase complex,
the same fluorophoric group can be used for each dNTP.
Alternatively, fluorescent dideoxy chain terminators
can be used in competition with unlabelled dNTP's,
under conditions where the number of terminated chains
20   is not so large as to deplete the number of
unterminated chains to the point where fluorescence
increments associated with chain termination become
difficult to detect.   Each dNTP is presented with its
corresponding fluorescently-labelled ddNTP to the
25   template-primer-polymerase complex, and a single
fluorophore is sufficient.
(iii)   Absorption spectroscopy
         The direct measurement of template copy
growth by detecting the increase in ultraviolet light
30   absorption at approximately 250-260nm caused by
addition of new nucleotides to the copy strand is not
sensitive.   However, the availability of relatively
large amounts of starting material (template) through
DNA cloning or polymerase chain methods, allied to
35   powerful U-V laser sources such as frequency-tripled
Nd-Yag lasers, makes it technically possible to measure

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template-copy growth directly by absorption
spectroscopy.

        The advantages of these optical methods over
existing methods are very large. They include:-

5   -   potential for high speed (e.g. 10 bases/sec)
    -    miniaturisation and parallel flow lanes
    -    no need for gel electrophoresis
    -    direct signal transfer to computer
    -    no need for radioactive nucleotides
10 -   greater chance for complete automation due to
         absence of gel separation steps.

        The following Examples illustrate the
invention.

15 EXAMPLE 1
Detection of Polymerase Catalysed Nucleotide Addition
on a Silver Slide using Surface Plasmon Resonance

Materials and Methods
20 Reagents
    1.   0.25pmole/µl solutions of hybrid DNA (consisting of
    a 17 nucleotide primer sequence hybridised to a 20, 27 or a
    97 nucleotide template sequence) prepared in 10 mM
    phosphate buffer pH 7.4 containing 10mM phosphate
25 buffer pH 7.4 containing 10mM MgCl$_2$ and 50mM NaCl.
    2.   10mM phosphate buffer pH 7.4 containing 10mM MgCl$_2$
    and 50mM NaCl (sequencing phosphate).
    3.   10mM phosphate buffer pH 7.4.
    4.   DNA Polymerase 1 "Klenow fragment" diluted in
30 sequencing phosphate containing 250µM dATP, dCTP, dGTP
    and dTTP.
    5.   Block solution of 0.05% BSA in sequencing
    phosphate.

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Equipment

Rig

A single beam direct reference rig using a
632.8nm wavelength helium neon laser which interrogated
two spots and subtracted one signal from the other, was
used.

Pump and flow cell

A modified step motor pump which held 2
syringes was used to deliver different or identical
solutions to the 2 segregated areas of the flow cell.

Silver Slides

Silver coating thickness on the slides was
greater than that normally used for SPR work i.e. 60nm
to achieve an SPR width of approximately 0.6 degrees at
approximately 5% from the reflectance minimum.

Slide Tolerance Limits

The slides were rejected if the SPR profiles
on both areas of the silver slide were not similar in
shape and minimum.

Experimental Procedure

1. 1ml 10mM phosphate buffer ph 7.4 was added at
4μl/second to the silver slide.

2. 1ml sequencing phosphate was added at 4μl/second.

3. 1ml of hybrid (17/20 was used as the reference DNA)
17/27 or 17/97 was added at 2μl/second followed by 3
washes in sequencing phosphate.

4. 1ml of BSA block solution was added at 2μl/second
followed by 3 x 0.3ml washes in sequencing phosphate.

5. 1ml of Klenow plus nucleotides was added at
2μl/second followed by 3 x 0.3ml washes in sequencing
phosphate.

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Angle and time scans were obtained at strategic points during the experiment. The overall shift difference caused by the addition of extra nucleotides (3 for the reference, 10 for the 17/27 and 80 for the 17/97) was recorded.

Results

Hybrid	Hybrid coverage moles/mm^2	Nucleotide added moles/mm^2	SPR shift % reflectivity
17/97	3×10^{-15}	2.3×10^{-13}	3
17/27	Not determined	Not determined	2

The figures in the last column are additional changes over and above those of the 17/20 reference.

Conclusion

The SPR signal obtained from the addition of ten nucleotides to a primer-template complex by the Klenow fragment of DNA polymerase could be measured above the signal obtained from a three nucleotide addition in the reference sample. This clearly demonstrates the detection by SPR of the addition of seven nucleotides.

If the following change is made to the equipment then single nucleotide addition will be detected by SPRS.

The experiments were conducted by recording

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one reflectivity measurement per second. This gives
signal to noise resolution down to seven nucleotides.
However by increasing the measurement rate to 1000 per
second an improvement by a factor of 30 can be achieved
5   which will permit detection of single nucleotide
addition. Noise is proportional to the square root of
the number of readings taken, therefore the square root
of 1000 equals 30.

10   <u>EXAMPLE 2</u>
<u>Polymerase Catalysed Single Nucleotide Addition to</u>
<u>template DNA Immobilised on Silver Slides</u>

<u>Method</u>
15   1.   Template-primer DNA (a 17 nucleotide primer
annealed to a 97 nucleotide template) was immobilised
on the silver slides at a coverage of 1.25×10^{-13}
moles/silver slide.
2.   The 4 nucleotide / polymerase mixes were made up as
20   follows in 10mM phosphate buffer pH 7.4 containing 10mm
$MgCl_2$ and 50mm NaCl:-
a)   5μM deoxyadenosine 5'-triphosphate + 200U/ml Klenow
fragment (Amersham T2141Z),
b)   5μM deoxycytidine 5'-triphosphate + 200U/ml Klenow
25   fragment,
c)   5μM deoxyguanosine 5'-triphosphate + 200U/ml Klenow
   ·   fragment,
d)   5μM deoxythymidine 5'-triphosphate + 200U/ml Klenow
fragment,
30   To each mix 100μCi/ml of the corresponding
^{32}P labelled deoxynucleotide triphosphate was added
(Amersham PB204, 205, 206 and 207).
        The mixtures a-d were added singly in the
sequence in which they appeared in the template strand
35   as follows:-

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Single nucleotide additions

1. The silver slide was placed on the biosensor hemicylinder and its position marked. The flow cell was placed on top of the slide.

5   2. 3ml of 10mM phosphate buffer pH 7.4 was flowed over the silver slide.

3. 1ml of 10mM phosphate buffer pH 7.4 containing 10mm $MgCl_2$ and 50mm NaCl was flowed over the silver slide.

4. 100µl of the nucleotide / enzyme mix was injected

10   and left on the silver slide for 5 minutes at room temperature.

5. The silver slide was washed with 5ml of 10mM phosphate buffer pH 7.4 containing 10mm $MgCl_2$ and 50mm NaCl + 0.005% Tween 20.

15   6. The silver slide was removed and blotted to remove any surface fluid then counted on the tritium channel of a scintillation counter.

        Incorporation of the deoxynucleotide was determined from the increase in counts on the silver

20   slides and calculated from the specific activity of the nucleotide/polymerase mixture used at that step.

        The results are shown in Table 1.

7. The silver slide and flow cell were replaced on the biosensor in the same position and steps 3-6 were

25   repeated with the next nucleotide/polymerase mixture until the predetermined nucleotide addition sequence was completed.

        Control silver slides were subjected to the same nucleotide addition procedure.

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Results

<u>Correct Sequence Single Nucleotide Addition to Silver
Slide Immobilised DNA</u>

Addition Nos.	Nucleotide Added	Radioactivity (counts per minute)	Moles nucleotide added /slide
1.	T	127	7.5×10^{-15}
2.	G	253	1.3×10^{-14}
3.	T	651	2.3×10^{-14}
4.	G	702	5.3×10^{-15}
5.	A	925	1.8×10^{-14}

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Conclusion

The addition of successive single nucleotides
by the Klenow fragment of DNA polymerase 1 to this
primer template hybrid immobilised at the silver
5   surface can be observed with radioactively labelled
nucleotides.

In the absence of the primer template hybrid
no successive increase in nucleotide addition is
observed.

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CLAIMS

1.   A method of sequencing a single-stranded
nucleic acid chain, which method comprises:-
a)   providing a template-primer complex comprising the
single-stranded chain (the template) and at its 3'-end
a double stranded chain (comprising the primer), the
complex being attached at one end to a solid surface,
b)   presenting a single nucleotide or nucleotide
analogue to the complex in the presence of a polymerase
under conditions to cause an extension of the primer (a
polymerisation event) by addition of the nucleotide (or
analogue) to the 3'-end of the primer. if the nucleotide
(or analogue) is complementary to and can hybridize to
the next free nucleotide at the 5'-end of the template,
and
c)   repeating step b) successively using different
nucleotides or nucleotide analogues,
d)   the method being characterized by detecting a
change in the template-extended primer complex
resulting from each polymerisation event that occurs
during performance of steps b) and c).

2.   A method as claimed in claim 1, characterised
by detecting, directly and by spectroscopic means, each
polymerisation event that occurs during performance of
steps b) and c).

3.   A method as claimed in claim 2, wherein each
polymerisation event is detected by evanescent wave
spectroscopy.

4.   A method as claimed in claim 2, wherein each
polymerisation event is detected by attenuated total
reflection spectroscopy.

5.   A method as claimed in claim 2, wherein
fluorescent nucleotide analogues are used in steps b)

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and c) and each polymerisation event is detected by
total internal reflectance fluorescence spectroscopy.

6.   A method as claimed in claim 2, wherein each
polymerisation event is detected by surface plasmon
5   resonance spectroscopy.

7.   A method as claimed in claim 6, wherein high
refractive index nucleotide analogues are used in steps
b) and c).

8.   A method as claimed in claim 2, wherein
10   fluorescent nucleotide analogues are used in steps b)
and c) and each polymerisation event is detected by
fluorescence spectroscopy.

9.   A method as claimed in claim 2, wherein each
polymerisation event is detected by absorption
15   spectroscopy.

10.   A method as claimed in any one of claims 1 to
9, wherein the single-stranded nucleic acid chain is
DNA.

11.   A method as claimed in any one of claims 1 to
20   10, wherein a continuous flow of pulses of
deoxynucleotides (dATP, dCTP, dGTP, dTTP) or
ribonucleotides (ATP, CTP, GTP, UTP) or analogues of
these nucleotides, each separated by a wash pulse, is
passed over the complex attached to the solid surface.

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1/2

DNA SEQUENCING BY SPRS DETECTION OF TEMPLATE COPY GROWTH:
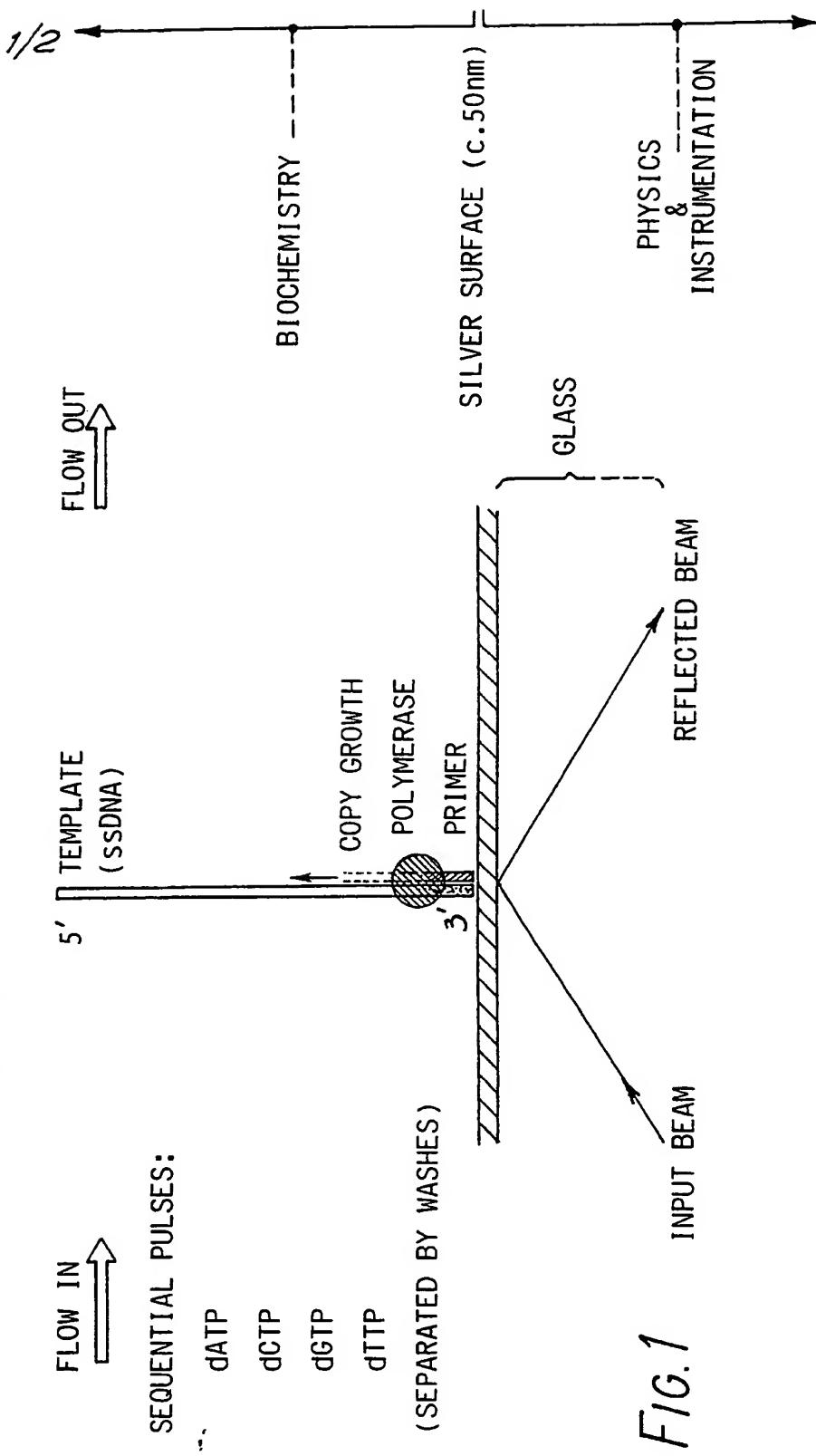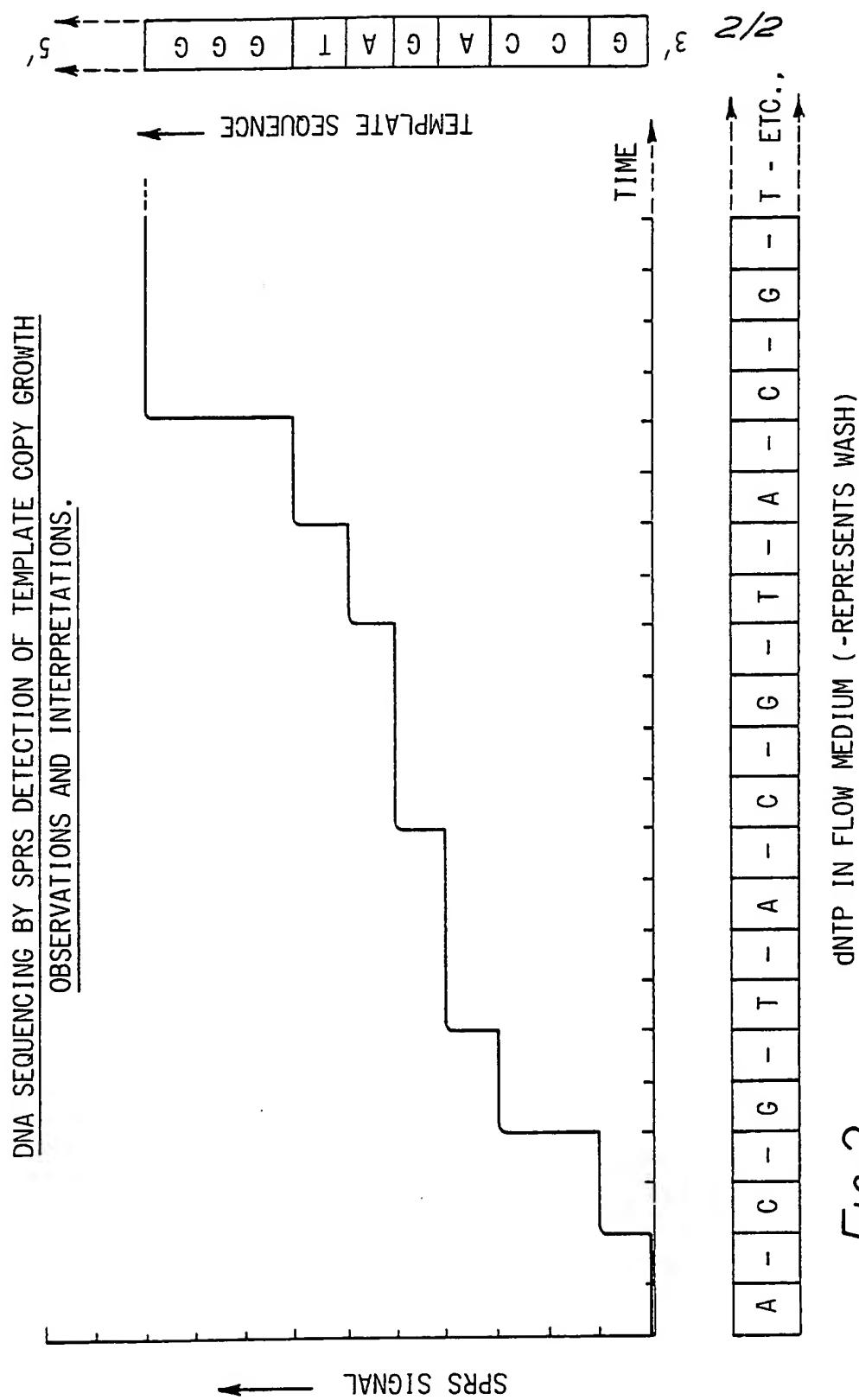
EXPERIMENTAL ARRANGEMENT

FLOW IN

SEQUENTIAL PULSES:

dATP

dCTP

dGTP

dTTP

(SEPARATED BY WASHES)

FLOW OUT

TEMPLATE
(ssDNA)

5'

COPY GROWTH

POLYMERASE

PRIMER

3'

BIOCHEMISTRY

SILVER SURFACE (c.50nm)

GLASS

PHYSICS
&
INSTRUMENTATION

REFLECTED BEAM

INPUT BEAM

FIG.1

2/2

DNA SEQUENCING BY SPRS DETECTION OF TEMPLATE COPY GROWTH
OBSERVATIONS AND INTERPRETATIONS.

TEMPLATE SEQUENCE

| G | C | C | A | G | A | T | G | G | G |

5' 3'

TIME

SPRS SIGNAL

dNTP IN FLOW MEDIUM (- REPRESENTS WASH)

| A | - | C | - | G | - | T | - | A | - | C | - | G | - | T | - | A | - | C | - | G | - | T - ETC., |

FIG.2

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC⁵: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System		Classification Symbols
IPC⁵	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	EP, A, 0223618 (NEW YORK MEDICAL COLLEGE) 27 May 1987 see column 4, lines 28-60; column 6, line 44 - column 7, line 14; column 15, line 34 - column 16, line 15 cited in the application --	1-10
Y	US, A, 4770992 (G.J. VAN DEN ENGH et al.) 13 September 1988 see column 7, line 60 - column 8, line 24 --	1-10
Y	EP, A, 0233053 (APPLIED BIOSYSTEMS) 19 August 1987 see page 2, lines 13-15; page 8, lines 13-30; page 22, line 1 - page 27, line 9 --	1-11
	./.	

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th July 1990	0 3 AUG 1990
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Mme N. KUIPER

Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO, A, 89/03432 (UNITED STATES DEPARTMENT OF ENERGY) 20 April 1989 see the whole document cited in the application	1-11

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 26/07/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0223618	27-05-87	US-A- 4863849 JP-A- 62085863	05-09-89 20-04-87
US-A- 4770992	13-09-88	None	
EP-A- 0233053	19-08-87	US-A- 4855225 JP-A- 62249049	08-08-89 30-10-87
WO-A- 8903432	20-04-89	None	